# Center for Veterinary Biologics
# and
# National Veterinary Services Laboratories
# Testing Protocol

## Supplemental Assay Method for Testing Growth-Promoting Qualities of Brain Heart Infusion Agar using *Bacillus subtilis* Spores and *Candida krusei* as Indicator Organisms

Date: July 13, 1999

Supersedes: March 9, 1978

Number: STSAM0902.01

Standard Requirement: 9 CFR, Part 113.25

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# 1. Introduction

## 1.1 Background

This is a Supplemental Assay Method (SAM) for testing Brain Heart Infusion Agar (BHIA) for growth-promoting qualities, as required in the Code of Federal Regulations, Title 9 (9 CFR), Part 113.25(b).

## 1.2 Keywords

Brain Heart Infusion Agar, *Bacillus subtilis*, *Candida krusei*, growth promotion

# 2. Materials

## 2.1 Equipment/instrumentation

**2.1.1** 30º-35ºC incubator

**2.1.2** 20º-25ºC incubator

**2.1.3** Sterile disposable cotton-plugged pipettes

**2.1.4** Sterile 10-ml disposable syringes with needles

**2.1.5** Biosafety cabinet

**2.1.6** Vortex mixer

## 2.2 Reagents/supplies

**2.2.1** Indicator Organisms: Use *Bacillus subtilis* (ATCC #6633) and *Candida krusei (*ATCC #6258) or equivalent organisms as specified in the current United States Pharmacopoeia (USP) as the control organisms in order to determine the growth-promoting qualities of the medium according to the 9 CFR, Part 113.25.

**2.2.2** Media: BHIA, with 500 Kinetic (Kersey) units of penicillinase per ml of media, screw-capped flasks, 250 ml in 500 ml, Soybean-Casein Digest Medium (SCDM), 9 ml in 16 x 125-ml screw-capped tubes. See **Section 9.1** for media formulations.

## 3. Preparation for the test

### 3.1 Personnel qualifications/training

The personnel doing the test must have experience or training in this protocol. This includes knowledge of aseptic biological laboratory techniques and preparation, proper handling, and disposal of biological agents, reagents, tissue culture samples, and chemicals. The personnel must also have knowledge of safe operating procedures and policies and Quality Assurance (QA) guidelines of the Center for Veterinary Biologics-Laboratory (CVB-L) or equivalent, as well as training in the operation of the necessary laboratory equipment listed in **Section 2.1**.

### 3.2 Preparation of equipment/instrumentation

**3.2.1** Turn on biosafety cabinets at least 1 hour before preparing positive control reagents or testing media for growth promotion.

**3.2.2** Monitor incubators daily for temperature according to the current version of GDOCSOP0004.

**3.2.3** Monitor freezers and coolers daily for temperature according to the current version of GDOCSOP0003.

## 4. Performance of the test

### 4.1 Establishing the dilutions to be used in testing new media.

**4.1.1** Remove a vial of the newly prepared stock culture from the freezer and rapidly thaw or rehydrate the culture with 1 ml of SCDM.

**4.1.2** Make tenfold dilutions of the stock cultures by using a 1-ml pipette to place 1 ml of the stock culture in 9 ml of SCDM ($10^{-1}$ dilution).

**4.1.3** Mix by inverting or vortexing the tube.

**4.1.4** Using a pipette, transfer 1 ml of $10^{-1}$ culture dilution in 9 ml of SCDM ($10^{-2}$ dilution). Mix as before and continue the procedure until the $10^{-10}$ dilution is prepared.

**4.1.5** Incubate the *B. subtilis* dilution tubes for 24 to 48 hr at 30°-35°C. Incubate the *C. krusei* dilution tubes for 2 wk at 20°-25°C.

**4.1.6** Examine the tubes visually for growth to establish the growth endpoint of each stock culture.

**4.1.7** Again make dilutions as before up to within 1 dilution of the growth endpoint **(Section 4.1.6)**. Using the last 3 dilutions, deposit 0.3 ml onto each of 2 petri dishes for each dilution.

**4.1.8** Pour 15-25 ml of Brain Heart Infusion Agar, with penicillinase, into each plate and swirl the plates to dispense the organisms. After the plates have solidified, incubate those containing *B. subtilis* for 7 days at 30°-35°C. Incubate the plates containing *C. krusei* for 14 days at 2O°-25°C.

**4.1.9** Count the number of colonies on all plates after the appropriate incubation period.

**4.1.10** Pick the dilution and the amount of inoculum (0.1-0.5 ml) which gives an average plate count of 20-60 colony forming units (CFU) and will be used for testing new media. Repeat **Sections 4.1.7 through 4.1.9** for 10 different vials of stock culture with 10 different batches of media, using these dilutions and inoculums.

**4.1.11** Due to a variation between batches of media and between vials of the standard organisms, a statistical tolerance is needed. The tolerances listed in Table I give the control limits for the average plate count which is found.

Table I. Control Limits for Single Batch of Media--Average of 2 Plate Counts

| **Average Plate Count in Preliminary Test** | **Control Limits** | |
|---|---|---|
| | ***bacillus*** | ***candida*** |
| 20 | 6 - 34 | 8 - 32 |
| 25 | 9 - 41 | 12 - 38 |
| 30 | 12 - 48 | 16 - 44 |
| 35 | 15 - 55 | 20 - 50 |
| 40 | 18 - 62 | 24 - 56 |
| 45 | 20 - 70 | 28 - 62 |
| 50 | 22 - 78 | 32 - 68 |
| 55 | 25 - 85 | 36 - 74 |
| 60 | 28 - 92 | 40 - 80 |

## 4.2 Testing the media

**4.2.1** Test each batch of BHIA prepared for sterility testing for growth-promoting qualities with the *C. krusei* and *B. subtilis* stock cultures. Thaw or rehydrate a tube of stock culture and make tenfold dilutions in SCDM. Transfer 1 ml of each thawed stock culture to 9.0 ml of SCDM ($10^{-1}$ dilution). Use a sterile pipette for each transfer.

**4.2.2** Mix the $l0^{-1}$ dilution by inverting the tube several times.

**4.2.3** Using a sterile pipette, transfer 1.0 ml of the $l0^{-1}$ culture dilution to 9.0 ml of SCDM ($l0^{-2}$ dilution). Mix as before and continue through the dilution established in **Section 4.1.10**.

**4.2.4** Deposit the established inoculum (for 20-60 CFU) of the dilution determined in **Section 4.1.10** into each of the 2 petri dishes for each of the stock organisms.

**4.2.5** Pour 15-25 ml BHIA with penicillinase into each of the 4 plates.

**4.2.6** Incubate the 2 petri dishes containing the *C. krusei* culture at 20°-25°C and count the number of colonies at the end of a 14-day incubation period.

**4.2.7** Incubate the 2 petri dishes containing *B. subtilis* culture at 30°-35°C and count the number of colonies at the end of a 7-day incubation period.

## 5. Interpretation of the test results

The average colony count per plate is determined for each organism and is compared with the control limits in Table I, **Section 4.1.11**, to see if this average falls within the limits expected. If the stock culture has not deteriorated and the average colony count is not within the control limits, the growth-promoting quality of that batch of media is in question and all tests with satisfactory (SAT) results will be reported as no tests (NT). All tests with unsatisfactory (UNSAT) results, when the growth-promoting qualities of the media are in question, will be repeated using a new batch of BHIA media.

## 6. Report of test results

Record the results of these growth promotion tests in the positive control log book next to the media control number for that batch of BHIA media.

## 7. References

**7.1** Code of Federal Regulations, Title 9, Part 113.25, U.S. Government Printing Office, Washington, DC, 1999.

**7.2** The U.S. Pharmacopeia, 1985, Vol. 21, pp 1151-1160, Mack Publishing Co., Easton, PA.

## 8. Summary of revisions

This document was rewritten from SAM 902, dated March 9, 1978, to meet the current NVSL/CVBL QA requirements, to clarify practices currently in use in the CVB-L, and to provide additional detail. The following are minor changes made from the superseded protocol:

**8.1** The *C. krusei* stock culture is now maintained lyophilized rather than frozen. The CVB-L has experienced less deterioration of the stock culture titer in the lyophilized state, but freezing is still an acceptable method of preservation.

**8.2** The inoculum size has been changed from a set 0.3 ml to a range of 0.1-0.5 ml to allow for better adjustment to the required 20-60 CFU.

**8.3** The NT and repeat test have been added to **Section 5**. The CVB-L will repeat only UNSAT tests. All SAT tests conducted with media with questionable growth promotion will be called NTs and will be judged on the results of the firm’s test.

# 9. Appendices

## 9.1 Media formulations

### 9.1.1 NVSL Media Formulation No. 10204

**BRAIN HEART INFUSION AGAR (BHIA)**

| | |
|---|---|
| Brain Heart Infusion Agar | 52 g |
| $QH_2O$ | 1000 ml |

Autoclave 20 min at 121°C.

### 9.1.2 NVSL Media Formulation No. 10423

**TRYPTICASE SOY BROTH (TSB)**
**or**
**SOYBEAN-CASEIN DIGEST MEDIUM (SCDM)**

| | |
|---|---|
| Trypticase Soy Broth | 30 g |
| $QH_2O$ | 1000 ml |

Autoclave 20 min at 121°C.

TSB and SCDM are 2 names for the same media formulation from different media companies.